JC07 Rec'd PCT/PTO 13 MAR 2002

FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: **03940005TA**

U S APPLICATION NO (IF KNOWN, SEE 37 CFR): **10/070920**

INTERNATIONAL APPLICATION NO	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US00/25186	**15 September 2000**	**16 September 1999**

TITLE OF INVENTION
IMPROVEMENT OF CLAVULANIC ACID PRODUCTION

APPLICANT(S) FOR DO/EO/US
THE JOHNS HOPKINS UNIVERSITY

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include itens (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☒ is attached hereto (required only if not communicated by the International Bureau).
   b. ☐ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☒ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☐ Certificate of Mailing by Express Mail
23. ☐ Other items or information:

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U.S. APPLICATION NO (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
10/070920	PCT/US00/25186	03940005TA

24. The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

		CALCULATIONS PTO USE ONLY
☐	Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1000.00**	
☐	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$860.00**	
☐	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$710.00**	
☐	International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**	
☒	International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**	
	ENTER APPROPRIATE BASIC FEE AMOUNT =	**$100.00**
	Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$130.00**

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	30 - 20 =	10	x $18.00	**$180.00**
Independent claims	7 - 3 =	4	x $84.00	**$336.00**
Multiple Dependent Claims **(check if applicable).**			☐	**$0.00**
	TOTAL OF ABOVE CALCULATIONS =			**$746.00**
☒	Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.			**$373.00**
	SUBTOTAL =			**$365.00**
	Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +			**$0.00**
	TOTAL NATIONAL FEE =			**$365.00**
☐	Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).**			**$0.00**
	TOTAL FEES ENCLOSED =			**$365.00**
			Amount to be: refunded	$
			charged	$

a. ☒ A check in the amount of **$365.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **50-2041** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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45,922

REGISTRATION NUMBER

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re patent application of Townsend et al.

International Number PCT/US00/25186 US Serial number: unassigned

Attorney Docket Number: 03940005aa

Filed: International Filing Date 15 September 2000

For: ***IMPROVEMENT OF CLAVULINIC ACID PRODUCTION***

STATEMENT TO SUPPORT FILING AND SUBMISSION IN ACCORDANCE WITH PCT Rule 13ter.2 and 37 C.F.R.§§ 1.821-1.825

Assistant Commissioner for Patents

Washington, D.C. 20231

Box SEQUENCE

Dear Sir:

In connection with a Sequence Listing submitted concurrently herewith, the undersigned states that:

1. the submission, filed herewith in accordance with 37 C.F.R.§ 1.821 (g), does not include new matter;

2. the content of the attached paper copy and the attached computer readable copy of the Sequence Listing, submitted in accordance with 37 C.F.R.§ 1.821(c) and (e), respectively, are the same; and

[redacted]

3. all statements made herein of their own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

Respectfully submitted,

Ruth E. Tyler-Cross

Reg. No. 45,922

March 13, 2002

Date

[redacted]

8/PRTS

10/070920

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IMPROVEMENT OF CLAVULANIC ACID PRODUCTION

This invention was made using funds from grants from the National Institutes of Health having grant number AI 14937. The government may have certain rights in this invention.

DESCRIPTION

BACKGROUND OF THE INVENTION

Field of the Invention

The invention generally relates to the enhancement of clavulanic acid production. In particular, the invention provides a method for increasing the production of clavulanic acid by: gene dosage with *orf2* from the clavulanic acid biosynthetic pathway in *Streptomyces clavuligerus*; and by manipulation of fermentation conditions, especially the concentration of D-G3P, a substrate of N2(carboxyethyl)arginine synthetase, the protein encoded by *orf2*. A method for preparing N2(carboxyethyl)arginine synthetase is also provided, as is an assay for identifying its substrates.

Background of the Invention

The β-lactams such as penicillin, cephalosporin and cephamycin were the first useful class of antibiotics discovered and are still in clinical use to combat infections. However, the extensive use of β-lactams has reduced their effectiveness due to the emergence of resistance among invading pathogens. There is therefore an increasing urgency to develop strategies aimed at overcoming acquired resistance.

Resistance to the antibiotics penicillin and cephalosporin is conferred upon bacteria via the β-lactamase enzymes. β-lactamases catalyze the hydrolysis of the antibiotics, rendering them inactive. One of the best examples of strategies aimed at overcoming acquired resistance is the development of β-lactamase inhibitors, exemplified by commercial products such as AUGMENTIN® and TIMENTIN®. These products are combinations of clavulanic acid, (a potent, naturally occurring β-lactamase inhibitor that is produced by the bacterium *Streptomyces clavuligerus*) together with other β-lactam antibiotics. As a result of this widespread clinical application, clavulanic acid production is currently valued in excess of a billion dollars.

With the advent of recombinant DNA technology and protein engineering, there has been increasing interest in manipulating biochemical pathways, especially those which produce commercially viable compounds. This may be accomplished, for example, by amplifying the genes encoding the rate-limiting enzymes of the pathway, or by modifying enzyme specificity. To effectively enhance productivity by genetic manipulation, identification of the controlling steps in the biosynthetic pathway is essential. Frequently an enzyme involved in the conversion of primary metabolites to the first dedicated intermediate in the biosynthetic pathway for the secondary metabolite has been identified as the rate-determining enzyme. Increasing the gene dosage of the rate-determining enzyme may result in the overproduction of flux-limiting enzyme and lead to increased production of the final product. This is the case, for example, in *Cephalosporium acremonium* and *Streptomyces clavuligerus* where the non-ribosomal condensation of the ACV tripeptide from α-aminoadipic acid, valine and cysteine is the rate-determining step in the penicillin pathway (Malmberg, L.-H., Hu, W.-S. and Sherman, D.H. **1993**, *J. Bacteriol.* **175**:6916-6924). However, for this approach to be viable, identification of all enzymes involved in the pathway is required, as well as a detailed kinetic analyses for each reaction step.

Clavulanic acid is produced by fermentation processes employing the bacterium *Streptomyces clavuligerus*. While the gene cluster responsible for clavulanic acid biosynthesis in this organism has been identified (Li, R.-F., Khaleeli, N. and Townsend, C.A. **2000**, *J. Bacteriol.* **182**:4087-4095), the complete biosynthetic pathway has not been sufficiently well-characterized to identify the rate-limiting reactions of the pathway. Therefore, it is currently not possible to rationally design recombinant DNA approaches to increasing clavulanic acid production. It would be highly desirable to elucidate the biosynthetic pathway of clavulanic acid and to utilize the information in order to enhance production of this clinically valuable compound by genetic manipulation.

SUMMARY OF THE INVENTION

The invention relates to the discovery that D-glyceraldehyde-3-phosphate is a primary metabolic precursor of clavulanic acid in an unusual thiamin pyrophosphate (TPP)-mediated reaction carried out by N2(carboxyethyl)arginine synthase. The enzyme is encoded by *orf2* in the producing organism, *Streptomyces clavuligerus*. Improvement of clavulanic acid production can be achieved by gene dosage and by the design/manipulation of fermentation conditions to attain favorable levels of D-G3P or L-arginine for synthesis. In particular, the invention provides a method for increasing the production of clavulanic acid by amplification of the *orf2* gene in an appropriate host, for example *S. clavuligerus* bearing

the intact pathway, and a rationale for improvements to fermentation conditions. The invention also provides a method for preparing the enzyme N^2-(2-carboxyethyl)arginine synthase; and an assay for detecting potential substrates of N^2-(2-carboxyethyl)arginine synthase.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Construction of plasmid pKC1139/pro-*orf2*-ter.

Figure 2. Fermentation of *S. clavuligerus* strains: *orf2* gene dosage study employing native promoter and replicating vector. ◆ = WT/pKC1139/pro-*orf2*-ter (in SA+ medium); ■ = WT (in SA+ medium).

Figure 3. Construction of integrative plasmid pSET152/pro-*orf2*.

Figure 4. Southern hybridization analysis of chromosomal DNA isolated from recombinant strains using *orf2* probe. Lane 1. WT/pSET152-EcoRI; Lane 2. WT/pSET152/ermE(XbaI)-*orf2*-EcoRI; Lane 3. WT/pSET152/pro-*orf2*-EcoRI/HindIII; Lane 4. WT/pSET152-EcoRI/HindIII; Lane 5. WT/pSET152/ermE(HindIII)-*orf2*- EcoRI/HindIII; Lane 6. 2-2-23/pSET152/ermE(HindIII)-*orf2*- EcoRI/HindIII.

Figure 5. Fermentation of *S. clavuligerus* strains: *orf2* gene dosage study employing native promoter and integration vector. ■ = WT/pSET152/pro-*orf2* (in SA+ medium);◆ = WT (in SA+ medium).

Figure 6. Construction of integration vector pSET152/*ermE*(XbaI)-*orf2*.

Figure 7. Construction of integration vector pSET152/*ermE*(HindIII)-*orf2*.

Figure 8. Fermentation of *S. clavuligerus* strains: *orf2* gene dosage study employing *ermE* promoter and integration vector.◆ = WT; ■ = WT/pSET152*ermE* (XbaI)-*orf2*; ● = WT/pSET152*ermE* (HindIII)-*orf2*.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

As illustrated in the Examples below, we have discovered that the enzyme encoded by *orf2* of the clavulanic acid gene cluster catalyzes the first biosynthetic reaction in the clavulanic acid pathway (Scheme 1). It mediates the condensation of two primary metabolites, D-glyceraldehyde-3-phosphate (D-G3P) and L-arginine, to give the first intermediate N^2-(2-carboxyethyl)-L-arginine (CEA). The level of Orf2 therefore controls the uptake of both precursors from primary metabolism to clavulanic acid production.

Although the genetic sequence of *orf*2 and the deduced amino acid sequence have been previously identified by Jensen et al. (Canadian Patent 2,108,113) the function of Orf2 that is suggested by Jensen et al. (acetohydroxyacid synthase-like activity, i.e. participation in the biosynthesis of branched chain amino acids) is incorrect. As shown in Example 1 below, the present disclosure provides the correct description of the enzymatic activity of the *orf*2 gene product, N^2-(2-carboxyethyl)arginine synthase, including elucidation of its substrates D-G3P and L-arginine.

Based on this discovery, we investigated whether an increase in *orf2* gene expression would increase the flux of precursors from the primary metabolite pool into the clavulanic acid pathway and improve clavulanic acid yield. It is known that the introduction and expression of homologous or heterologous gene(s) involved in antibiotic biosynthesis in a producer strain can be a way to enhance productivity , to affect percent composition of a desired final product, or to synthesize hybrid antibiotics. For example, a 40% increase in penicillin production was obtained by transforming *P. chrysogenum* with extra copies of a DNA fragment containing the genes involve in the last two steps of the pathway (Veenstra, A.E., P. van Solingen, R.A. L. Bovenberg and L.M.H. van der Voort, **1991**. *J Biotechnol.* 17:81-90.). Accordingly, as illustrated in the Examples below, we cloned *orf2* and its upstream regulatory sequence into both replicative and integrative vectors. The results showed that clavulanic acid production levels in recombinant strains increased by 53% and 68%, respectively. We also placed *orf2* under the transcriptional control of the constitutive and strong *ermE** promoter. The results showed that the recombinant strains produce 34% to

68% more clavulanic acid than the wild-type strain. Further, Southern hybridization showed that all recombinant strains constructed contained the additional copy of *orf2* integrated into the chromosome.

The present invention provides a method for increasing the production of clavulanic acid in *Streptomyces clavuligerus*. The method involves increasing the production of the enzyme N^2-(2-carboxyethyl)arginine synthase in the bacterium. In one embodiment of the present invention, the increase is effected by providing the bacterium with additional copies of the DNA that encodes the enzyme. By "additional copies of the DNA", we mean copies of the DNA that are introduced into the bacterium via genetic engineering techniques.

By "DNA that encodes the enzyme" we mean any sequence of nucleotides that encodes a functional N^2-(2-carboxyethyl)arginine synthase enzyme. Those of skill in the art will recognize that this may include the "native" enzyme (including any elements such as native control elements e.g. promoters, ribosome binding sites, terminators and the like) or many variations of the "native" enzyme, including but not limited to forms of the enzyme with conservative amino acid substitutions, non-conservative amino acid substitutions, insertions, deletions, truncations, fusions, and the like. The enzyme may be genetically engineered, for example, in order to maximize such parameters as substrate binding efficacy, rate of catalysis, stability, molecular weight, or to produce chimeric proteins, or to carry out directed evolution or gene shuffling, and the like. DNA encoding any suitable form of N^2-(2-carboxyethyl)arginine synthase may be used in the practice of the present invention, so long as the resulting form of the enzyme carries out the enzymatic reaction of the condensation of two primary metabolites, D-glyceraldehyde-3-phosphate (D-G3P) and L-arginine to give the first intermediate in the clavulanic biosynthetic pathway, N^2-(2-carboxyethyl)-L-arginine (CEA). The present invention is intended to encompass all such forms of the enzyme, as well as the DNA sequences which encode them.

The DNA sequence itself may be of any suitable sequence that encodes a functional form of the N^2-(2-carboxyethyl)arginine synthase enzyme. The DNA sequence may be modified in any of a variety of appropriate ways, including but not limited to: the introduction of restriction enzyme sites, manipulation of the sequence to facilitate cloning or handling of the DNA, to create chimeric forms of the protein, to effect alterations in the

amino acid sequence of the encoded enzyme, to increase or decrease stability of the DNA itself, or of the encoded enzyme. Such modifications may include various appropriate chemical modifications, the introduction of various control elements and manipulation of their location (e.g. promoters, ribosome binding sites, terminators), and the like. Any suitable form of DNA encoding a functional form of the N^2-(2-carboxyethyl)arginine synthase enzyme may be used in the practice of the present invention.

The production of N^2-(2-carboxyethyl)arginine synthase in *Streptomyces clavuligerus* is a controlled event. It may be controlled by positive or negative regulators of transcription, as well as other factors in the fermentation medium. Modulation of production may thus be controlled, for example, by the deletion of a copy of a negative regulator (e.g. a transcription factor) or by the insertion of additional copies of a positive relator (transcription factor), or by modifying the stress (fermentation) conditions in a manner that alters the activity or production of such factors. For example, a transcription factor that upregulates the transcription of *orf2* may be added to the fermentation medium.

The form of the N^2-(2-carboxyethyl)arginine synthase enzyme utilized in the methods of the present invention may be the native form of the enzyme, or may be any of various other modified forms of the enzyme. Examples of such modifications include but are not limited to post-translational modifications carried out within a host organism (e.g. acylation, glycosylation, phosphorylation, and the like), or *in vitro* modifications (e.g. chemical modifications, proteolytic modifications, labeling, attachment to a substrate, and the like). Any form of the enzyme that is competent to carry out the condensation of D-glyceraldehyde-3-phosphate (D-G3P) and L-arginine, to yield N^2-(2-carboxyethyl)-L-arginine may be used in the practice of the methods of the present invention. The enzyme may also be fused with another protein to generate a chimeric form of the enzyme.

In one embodiment of the invention, the DNA is provided to the bacterium by the introduction of a plasmid encoding the N^2-(2-carboxyethyl)arginine synthase gene. In a preferred embodiment of the present invention, the plasmid is the replicating plasmid pKC1139/pro-orf2-ter. However, those of skill in the art will recognize that many other plasmid vectors may also be utilized in the successful practice of the present invention. For example, plasmids such as pIJ680, pIJ702, pWHM1109, and pKC1218 may also be used.

Any suitable plasmid that provides within the bacterium a DNA sequence encoding an appropriate form of N^2-(2-carboxyethyl)arginine synthase may be utilized in the practice of the present invention.

In one embodiment of the present invention, the DNA that encodes the N^2-(2-carboxyethyl)arginine synthase gene is integrated into the host genome. The invention thus also provides a host cell in which the *orf2* gene is stably integrated. In one embodiment of the present invention, the integration is carried out utilizing an integrative vector which may, for example, be a site-specific integrative vector. In preferred embodiments, the site-specific integrative vectors are pSET152/pro-orf2, pSET152/ermE(XbaI)-orf2 or pSET152/ermE(HindIII)-orf2. However, those of skill in the art will recognize that other integrative vectors may also be used in the practice of the present invention, for example pOJ436, pOJ444 and pGM9. Any suitable integrative vector that results in the stable integration of a DNA sequence encoding an appropriate form of N^2-(2-carboxyethyl)arginine synthase may be utilized in the practice of the present invention.

In one embodiment of the present invention, the DNA sequences that encode N^2-(2-carboxyethyl)arginine synthase may include a promoter. The promoter may be the native promoter, or a promoter that has been genetically engineered into the DNA. The promoter may be a constituitive promoter and may be a promoter that is recognized by those of skill in the art as a strong promoter. In a preferred embodiment of the present invention, the promoter is the *ermE** promoter. However, those of skill in the art will recognize that many suitable promoters exist which may be used in the practice of the present invention, for example, *PtipA*, *aph* and *xyl*. Any suitable promoter that results in appropriate expression of DNA encoding N^2-(2-carboxyethyl)arginine synthase may be utilized in the present invention. In addition, the sequence of any given promoter may also be altered, for example, to provide ease of genetic manipulation, or to modulate the relative strength of the promoter.

In a preferred embodiment of the present invention, the bacterium which is utilized for enhanced production of clavulanic acid is *Streptomyces clavuligerus*. However, those of skill in the art will recognize that other host organisms may also be utilized in the practice of the present invention. For example, other *Streptomyes* such as *S. lividans*, *S. coelicolor*, *S. jumonjinensis* (e.g. ATCC 29864), *S. lipmanii*, *S. katsurahamanus* (e.g. strain T272), *S.*

parvulus, *S. griseofulvus*, and *S. antibioticus*. In addition, host organisms may not be limited to bacterial hosts but may include other expression hosts such as yeast, plant cells, or cultured cells. Any host capable of carrying out the biosynthesis of clavulanic acid may be utilized in the practice of the present invention.

The invention further provides a method to increase clavulanic acid production by effecting alterations in the bacterial growth conditions, e.g. precursor concentration, fermentation conditions, additives such as dihydroxyacetone, glycerol, inositol and glucuronate, etc. Those of skill in the art will recognize that many such parameters can be altered and all such variations are intended to be within the scope of the present invention. For example, the concentration of the substrates utilized by the enzyme N^2-(2-carboxyethyl)arginine synthase (D-glyceraldehyde-3-phosphate and L-arginine) may be increased. This may be accomplished in any of a variety of ways, including but not limited to: the addition of those substances or precursors of those substances to the growth medium, either directly, or by the introduction of or genetic manipulation of genes which, either directly or indirectly, enhance their production; and effecting a decrease in the breakdown of the substrates (e.g. by reducing the activity of glyceraldehyde dehydrogenase), or modulating the diversion of the substrates into other pathways, for example by modulating other enzymatic pathways in which they participate. In addition, the availability of TPP may be increased. Other fermentation conditions such as temperature, ionic strength, nutrient levels, and the like may also be altered. Any fermentation parameter which has the effect of increasing the concentration of, or increasing the activity of the enzyme N^2-(2-carboxyethyl)arginine synthase, may be altered in the practice of the method of the present invention.

The present invention also provides a method for preparing the enzyme N^2-(2-carboxyethyl)arginine synthase. In general, the method comprises the steps of culturing and harvesting a host cell which synthesizes the enzyme, obtaining an appropriate enzyme-containing fraction from the host (for example, the supernatant after a centrifugation step), subjecting the fraction to ammonium sulfate precipitation, resuspending the precipitated protein pellet, and chromatographing the resuspended protein on an affinity column. In a preferred embodiment of the present invention, the ammonium sulfate precipitation utilized

is a 30% precipitation. In a preferred embodiment of the present invention, the affinity column is an L-arginine agarose affinity column.

In a preferred embodiment of the present invention, the cells were lysed in 50 mM N-[2-acetamido]-2-iminodiacetic acid (ADA) pH 6.0, 5 mM $MgCl_2$, 0.5 mM thiamine pyrophosphate, 1 mM DTT, 2 mM EDTA and 12.5 ug/mL Trypsin Chymotrypsin Inhibitor. This buffer, ADA pH 6.0, was chosen so as to approach the theoretical pI (5.1)of the protein without adversely affecting the activity in preparation for an isoelectric precipitation (the salting out of proteins at pHs near their theoretical pIs). Cellular debris was removed by centrifugation and finely ground ammonium sulfate was added to the cell free extract to a final concentration of 30%. The protein solution was incubated on ice for 15 min with stirring. Centrifugation at 13,000 x g yielded a protein pellet which was resuspended in 1 mL buffer [25% glycerol, 100 mM TrisHCl pH 8.0, 5 mM MgCl2, 12.5 ug/mL Trypsin Chymotrypsin inhibitor, 0.5 mM TPP and 1 mM DTT with rotary shaking on ice. Two distinctive features of this precipitation are the pH of the precipitation and resuspension buffers and their glycerol contents.

In a preferred embodiment of the preparation method, the host cell is *Escherichia coli*. However, those of skill in the art will recognize that any host cell capable of producing N^2-(2-carboxyethyl)arginine synthase may be utilized in the method of the present invention.

The present invention also provides an assay for the identification of potential substrates of N^2-(2-carboxyethyl)arginine synthase. Potential substrates include those which result in novel products (i.e. products which are not CEA) which could undergo biotransformation novel forms of antibiotics. The substrates may be naturally occurring or synthetic. For example, by modifying either the substrate L-arginine or D-G3P at atoms which are not directly involved in the enzymatic reaction, it is possible to retain the ability of those substrates to be acted upon by the enzyme, and produce a condensation product that is not CEA but which includes the modification. Examples include the attachment of a moiety of interest to the side chain of arginine, such that the moiety is retained in the reaction product. The resulting condensation product may go on to be acted on by other enzymes and thus produce other novel substances, or the product may be desirable in its own right.

The assay is carried out by incubating a potential substrate with the enzyme in the

presence of TPP and one known substrate and detecting the presence or absence of a condensation product that is produced. If the result is positive (i.e. if a condensation product is detected) then the putative substrate may be deemed a substrate of the enzyme. If the potential substrate is intended to replace or mimic arginine, then the known substrate may be D-G3P. Conversely, if the potential substrate is intended to replace or mimic D-G3P, then the known substrate may be arginine. In a preferred embodiment of the present invention, the known substrate may be labeled, for example by radiolabeling, (e.g. [U-^{14}C]Arginine. The label would then be incorporated into the product so that the product is readily detectable. However, those of skill in the art will recognize that many ways of designing such as assay exist. For example, the product may be detected by utilizing other detectable labels on the known substrate which would be incorporated into the product during the reaction, or monitoring the production of the product by some other method (e.g. HPLC). All such variations are intended to be encompassed by the assay of the present invention.

Further, the process described in the assay procedure may also be utilized in order to create novel condensation products. According to this facet of the invention, the enzyme may be utilized to condense any substances which are capable of acting as substrates for the enzyme in order to produce condensation products.

The present invention also encompasses a method for increasing the production of N^2-(2-carboxyethyl)arginine in a host cell by enhancing a rate of condensation of the substrates L-arginine and D-G3P. The enhancement may be effected by the enzyme N^2-(2-carboxyethyl)arginine synthase, for example by increasing the copy number of the synthase in the host cell.

The following Examples are included by way of illustration but should not be interpreted to limit the invention in any way.

EXAMPLES

EXAMPLE 1. Origin of the β-lactam Carbons in Clavulanic Acid from an Unusual Thiamine Pyrophosphate-Mediated Reaction

The primary metabolic precursors of clavulanic acid are known to be arginine [1,2] and a C3-intermediate thought to be derived from glycolysis.[3] Identification of the latter has defied assiduous investigation over many years. [4, 5] The first gene of the clavulanic acid gene cluster in *Streptomyces clavuligerus* encodes a thiamine pyrophosphate (TPP)-dependent enzyme that carries out the unprecedented condensation of L-arginine with D-glyceraldehyde-3-phosphate (**2** in Scheme 2) to give N^2-(2-carboxyethyl)arginine (**3** in Scheme 1, CEA), the first dedicated intermediate in clavulanic acid biosynthesis.[6]

Detailed isotopic labeling experiments have placed strict constraints on the mechanism of the coupling reaction that links the C_3- and C_5-building blocks drawn from primary metabolism to initiate clavulanic acid biosynthesis. It is known that H-2 of both glycerol (**4** in Scheme 2, H_E),[4, 7] and glyceric acid (**5** in Scheme 1, H_F) [5, 8] are lost on incorporation into clavulanic acid. Of the four remaining glycerol hydrogens (**4** in Scheme 2, $H_{A\text{-}D}$), only one is retained in **1**.[4] The identity of this single hydrogen (H_B) was determined in a stereochemical experiment in which only the *pro-(R)* arm of glycerol was radiolabeled specifically at one or the other diastereotopic methylene locus (**4**, H_A or H_B).[9] This telling result implied that, since stereochemical information is retained through the intermediates of glycolysis as far as phosphoenol pyruvate (PEP), the biosynthetic pathway must proceed in such a way to transmit this information to clavulanic acid (**1**). The suggested intermediacy of lactate[6] or pyruvate, [8, 10] therefore, can be excluded; that is, isotopic labels which are diastereotopic in **4** and **5** become achiral in a methyl group and, consequently, lose their ability to transfer label stereospecifically to clavulanic acid.

Two further observations made it possible to establish the stereochemical course of N—C bond formation in the construction of CEA (**3**). First, it could be shown that both H_A and H_B in glycerol (**4**) are completely retained in the formation of proclavaminic acid (**6** in Scheme 2).[11] Second, incubation of **5** stereospecifically labeled at C-4' (**5**, H_A or H_B) with clavaminate synthase demonstrated that the oxidative cyclization/desaturation to clavaminic

acid (7 in Scheme 2) occurred with clean stereochemical retention.[12] Knowing H_B survives the striking ring inversion to clavulanic acid (**1**), a complete stereochemical correlation could now be deduced as outlined in Scheme 2. It may be concluded that the C—O bond in **4** and **5** is replaced by the N—C bond in CEA (**3**) with overall retention of configuration. In sum, these results limit the possible precursor from primary metabolism to a C_3-carbohydrate likely lying between glycerol and PEP whose hydroxymethylene oxidation state is maintained throughout stereospecific CEA formation.

Identification of the 3-carbon unit became possible with the recent discovery of a new biosynthetic enzyme which cyclizes CEA (**3**) to the β-lactam ring contained in proclavaminic acid (**6** in Scheme 2). This ATP/Mg^{++}-dependent protein catalyzes a previously unknown reaction type, a β-lactam synthetase, and is encoded by the second gene in the clavulanic acid biosynthetic cluster.[13, 14] The first gene of the cluster lies directly upstream and gives rise to a protein of translated molecular mass 60, 907 Da showing sequence identities as high as 29% to acetolactate synthases from several sources, and to a lesser extent to pyruvate oxidases. The potential relation of a thiamine pyrophosphate-dependent enzyme such as these to any step in clavulanic acid biosynthesis was not obvious.

To examine the biosynthetic role of its encoded gene, *orf2* was cloned into pET24a (Novagen) and used to transform *E. coli* B834(DE3). As a control, this host was also transformed with the vector alone. The recombinant clones were separately inoculated into LB medium and, once growth had reached $A_{600} = 0.7$, they were transferred to sterile flasks and induced with IPTG. After 3 h, 1 mM [U-^{14}C]arginine (50 μCi/mmol) was added and incubation was continued for an additional 21 h at 28 °C. The cells were harvested by centrifugation and the supernatants were analyzed by HPLC after microfiltration [Spherex 18 5μ ODS(4) (Phenomenex), 50 mM ammonium bicarbonate as eluant]. The appearance of radioactivity in the chromatograms was monitored by scintillation counting. Significant radioisotope was detected in samples with a retention time coincident with CEA in the sample from the recombinant bearing *orf2*, but not the control culture. This finding implied that the over-produced protein encoded by the first gene of the biosynthetic cluster catalyzed the condensation of L-arginine with some primary metabolite available in *E. coli* to synthesize CEA (**3**), that is, the elusive C_3-unit itself.

Preliminary identification of the precursor of the C_3-unit was sought in an *in vitro* experiment. A cell-free extract (CFE) was prepared from frozen cell paste of the recombinant strain according to the method of Busby et al.[15] [U-^{14}C]Arginine was incubated in the presence of various potential C_3-intermediates (30 mM), TPP (1.5 mM) and the CFE in Tris buffer. The glycolytic intermediates examined were several phosphoglyceric acids (PGA, Table 1), D,L-glyceraldehyde-3-phosphate (D,L-G3P), dihydroxyacetone phosphate (DHAP), phosphoenolphosphate (PEP), D,L-glyceraldehyde (D,L-GA) and pyruvic acid (PA). After 3 h reaction at room temperature, the protein was removed by membrane filtration (Ultrafree 5000, Millipore) and the samples were analyzed by HPLC and scintillation counting as before. Despite our fear that extensive equilibration among the intermediates of glycolysis would cloud the outcome, the results of this experiment were clear, if unexpected. Although low levels of radioactivity appeared in CEA in every trial, the most efficient production of this first biosynthetic intermediate was observed with DHAP (Table 1).

As a first step toward purifying CEA synthase, it was discovered that a fortuitously efficient precipitation of the over-produced enzyme could be carried out with 30% ammonium sulfate. The protein pellet was resuspended and dialyzed[16] to give substantially pure CEA synthase (>95%) when examined by SDS-PAGE. Rescreening of the glycolytic intermediates with the partially purified enzyme gave reduced background counts in CEA and appeared to confirm the identity of DHAP as the precursor of the β-lactam carbons of clavulanic acid from primary metabolism (Table 1). A large-scale incubation of DHAP and L-arginine provided further evidence of this remarkable reaction yielding a single product whose chromatographic behavior and ^{1}H-NMR spectrum were identical to an authentic specimen of CEA (**3**).[13]

However, re-evaluation of D,L-G3P at higher concentration (60 mM) gave a greater incorporation in to CEA (**3**) (Table 1). Suspecting that one or the other enantiomer of G3P might be inhibitory, D-G3P was generated and found to give a conversion to CEA comparable to that of DHAP. It now appeared, therefore, that both DHAP and D-G3P could serve as substrates for the enzyme in the synthesis of CEA.

Triosephosphate isomerase (TIM) mediates the isomerization of DHAP and G3P and

is notorious for its exceptionally high catalytic activity.[17] Even a slight contamination by this enzyme could be responsible for the DHAP/D-G3P interconversion apparently carried out by CEA synthase. To examine this possibility, the substantially pure solubilized 30% ammonium sulfate pellet was loaded onto an L-arginine-agarose affinity column (Sigma) and eluted with a gradient of NaCl to give a highly purified sample of CEA synthase as judged by SDS-PAGE. The conversion of DHAP to D-G3P in a standard TIM assay[18] carried out in presence of the affinity-purified synthase, but in the absence of L-arginine, was significantly less efficient (<0.1%) than that from the 30% ammonium sulfate pellet (*ca.* 20%). Importantly, while the specific activities of the time-dependent transformation of D-G3P + [^{14}C]-L-arginine to CEA (**3**) in the presence of TPP increased as the purification of the protein advanced, the conversion of DHAP, while initially high in the CFE, fell successively to background when assayed with the affinity purified enzyme. Another round of purification through the affinity step gave homogeneous protein by SDS-PAGE and essentially unchanged activities for these two substrates indicating the trace TIM activity had been removed.

Thiamine pyrophosphate is absolutely required for enzyme activity, in keeping with the translated signature motif noted in Orf2. This is an unusual transformation for this cofactor more commonly associated with C–C bond breaking and bond-forming reactions as, for example, transketolases or the decarboxylation of α-ketoacids. CEA synthase mediates an internal redox reaction and a β-elimination/addition leading to N–C bond formation in the synthesis of **3**. This is a pleasingly adroit process in which the carboxyethyl of the product **3** required for β-lactam formation is generated by the capture of a glycolytic intermediate having the equivalent oxidation state.

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Example 2. Enhancement of clavulanic acid production by amplification of the *orf2* gene cloned into a replicating plasmid:

There is a 495-bp intergenic region between *orf1* and *orf2*. *Orf1* is transcribed divergently to *orf2*, so this region must contain the regulatory sequence that controls the expression of *orf2* and other downstream biosynthetic genes. To ensure that *orf2* cloned on a plasmid will be regulated the same way as the wild-type chromosomal copy of the DNA, *orf2* and its upstream region (2216 bp) were amplified by PCR. A 400-bp DNA fragment containing a terminator sequence that was originally from *fd* virus was excised from *Streptomyces* plasmid pIJ486 (18) and inserted into the *Eco*RV-*BamH*I digested and T_4 DNA polymerase treated bifunctional vector pKC1139. The *orf2* PCR product was inserted into the *Hind*III site to give rise to the final recombinant plasmid (Figure 1). This plasmid was transformed into wild-type *S. clavuligerus* by protoplast transformation (19). A standard fermentation in regular SA medium (13) containing 1% arginine and 1% glycerol (SA+) was carried out in shake flasks. Samples were taken at different time points during the fermentation and standard imidazole assay was perfomed (6) (16). Cell densities measured at 600 nm (OD_{600}) showed that all cultures grew at similar rates. Figure 2 shows the results from the imidozale assays. *S. clavuligerus* containing pKC1139/*pro-orf2-ter*. produced about 55% more clavulanic acid than wild-type *S. clavuligerus*. Thus, this genetic modification provides a viable means for increasing production of clavulanic acid.

Example 3. Improvement of clavulanic acid yield by using a site-specific integrative vector.

Because plasmids often cause pleiotropic effects on antibiotic production and on the developmental life cycle of producing strains, in some cases the presence of replicating plasmids can considerably reduce antibiotic production (3). To investigate whether this is the case for an *orf2* gene dosage study in *S. clavuligerus*, we decided to use the integration method to insert a additional copy of *orf2* into chromosomal DNA.

ΦC31 is a broad-host-range temperate streptomycete phage. ΦC31 derivatives can integrate into many different *Streptomyces* spp., including *S. clavuligerus*. ΦC31 vectors

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containing an *oriT* element have shown a consistently high transformation efficiency (1.5×10^5 to 3.8×10^6 in *S. ambofaciens*) as well as a unique integrative site (*attB*) on the chromosome. Plasmids or cosmids that integrate site-specifically at the ΦC31 attachment sites give rise to stable exconjugants (10). They can be propagated without detectable loss of plasmid markers, even in the absence of drug selection (10). Interestingly, ΦC31-driven, site-specific recombination is apparently very efficient since it was reported that plasmids containing as much as 8-kb of homologous DNA were found only at the ΦC31 attachment site, with no detectable integration by homologous recombination (5). This discovery is very important for our study because the constructed recombinant plasmid for *orf2* will contain both a homologous DNA fragment and site-specific integration elements. The preference for site-specific integration will generate strains containing an additional copy of *orf2* inserted site-specifically on chromosomal DNA.

pSET152 is a bifunctional integration plasmid derived from ΦC31 (10). The PCR product of *orf2* and its native promoter sequence was inserted into the *Eco*RVsite of pSET152 to give *orf2* integration vector pSET152/pro-orf2 (Figure 3). This plasmid was introduced into both wild-type and an *orf2* disruption mutant of *S. clavuligerus* (1) (11). The chromosomal DNA isolated from one apramycin resistant transformant was digested with *Eco*RI-*Hind*III and then hybridized with an *orf2* probe. As shown in Figure 4 (lanes 3 and 4), 12-kb and 1.8-kb hybridization bands indicate that there are two copies of *orf2* in the chromosome, one is the wild-type copy and the other one is the integrated copy cloned on pSET152/pro-orf2. Only the 12-kb band was observed in DNA isolated from *S. clavuligerus* transformed with pSET152 indicating the vector has site-specifically integrated into the chromosome.

The integrated copy of *orf2* could restore clavulanic acid production in the *orf2* disruption mutant, indicating that the integrated *orf2* was functional (data not shown). Fermentation in SA+ medium showed that the recombinant strain produced about 66% more clavulanic acid than wild-type strain when the growth rate was identically maintained (Figure 5).

Example 4. Expression of the additional *orf2* gene with the *ermE promoter in *S. clavuligerus*.**

We also used the constitutive and strong *ermE** promoter (4) to investigate the effect of a different promoter on clavulanic acid production in *S. clavuligerus*. *orf2* was excised from its *E. coli* overexpression vector pET24a/orf2-orf3 along with the ribosome binding sequence and placed downstream of the *ermE** promoter in pIJ4070. To obtain maximal expression, *orf2* was inserted into two different sites (*Xba*I and *Hind*III) downstream of *ermE** promoter to generate a 49-bp and a 70-bp region between the −10 sequence and translation initiation codon. A DNA fragment containing the *ermE*-(Xba*I*)-orf2* or *ermE*-(Hind*III*)-orf2* cassette was cloned into pSET152 to generate two integration vectors, pSET152/ermE(XbaI)-orf2 and pSET152/ermE(HindIII)-orf2 (Figures 6 and 7). The recombinant derivatives were introduced into both the *orf2* disruption mutant, and wild-type of *S. clavuligerus* where integrtion was successfully observed..

Chromosomal DNA isolated from *S. clavuligerus* transformed with pSET152 or pSET152/ermE(XbaI)-orf2 was digested with *Eco*RI, while chromosomal DNA isolated from *S. clavuligerus* transformed with pSET152 or pSET152/ermE(HindIII)-orf2 was digested with *Eco*RI-*Hind*III. Southern hybridization with an *orf2* probe showed that strains transformed with recombinant derivatives gave two positive bands (12 kb and 2.2 kb), which correspond to the wild-type copy and the integrated copy of *orf2* on the chromosome, respectively, whereas, strains transformed with pSET152 gave only a 12-kb positive band corresponding to the wild-type *orf2* from the clavulanic acid gene cluster (Figure 4, lanes 1 and 5).

To examine the effect of the *ermE** promoter on the expression of the additional copy of *orf2* and on clavulanic acid production, production levels of antibiotic in SA+ liquid cultures of the wild-type and recombinant strains were determined. Fermentation in shake flasks showed that the *orf2* disruption mutant could be complemented by the integrated *orf2*, indicating the expression of *orf2* under the transcriptional control of *ermE** promoter (data not shown). Wild-type *S. clavuligerus* harboring pSET152 gave the same level of clavulanic acid production as the wild-type strain, indicating that the integration of pSET152 has no negative effect on clavulanic acid production (data not shown). Shake flask fermentation of

both *S. clavuligerus* (pSET152/ermE(XbaI)-orf2) and *S. clavuligerus* (pSET152/ermE(HindIII)-orf2) gave increased yield of clavulanic acid, but the former produces about 34% greater antibiotic and the later gives about 68% more (Figure 8), indicating that the larger separation between the initiation codon and the *ermE** promoter is more efficient for *orf2* expression.

Example 5. Stability of integrative vectors pSET152/pro-orf2, pSET152/ermE(XbaI)-orf2 and pSET152/ermE(HindIII)-orf2 in *S. clavuligerus*

The stability of all pSET152-derived vectors was tested in their *S. clavuligerus* transformants. The apramycin resistant colonies on the primary plates were transferred onto slants absent selective pressure. After growing for 5 days, a seed medium was inoculated and grown for 72 h and followed by 120 h or 144 h fermentation, both also carried out without selective pressure. Total DNA was isolated from 120 h or 144 h fermentation cultures and transformed *E. coli* DH5α cells. No apramycin resistant colonies were observed on any of the transformation plates, indicating that there are no free replicating plasmids in these strains after three generations of growth without antibiotic selection.

EXPERIMENTAL

Bacterial strains, vectors and growth conditions.

Streptomyces clavuligerus ATCC27064, *Escherichia coli* JM110, *Escherichia coli* DH5α were obtained from the American Type Culture Collection, Manassas, VA.

S. clavuligerus was maintained on SP medium (per liter): yeast extract, 10 g; glycerol, 10 g; Bacto-agar, 20 g; pH 6.8. Seed medium consisting of tryptic soy broth (Difco; Detroit, MI) was inoculated with spores of *S. clavuligerus* and grown at 28 °C on a rotary shaker (300 rpm) for 72 h. For clavulanic acid production mycelia from the seed cultures were inoculated into SA medium (13) plus 1% arginine and 1% glycerol at 5%, and this culture was grown under the same conditions as the seed culture. *Escherichia coli* strains were grown in either Luria broth, or TB broth as liquid medium or agar plates (17). The cloning vectors pIJ486, pKC1139 and pSET152 were provided by C. R. Hutchinson (University of Wisconsin,

Madison, WI). The cloning vector pT7Blue-3 and the expression vector pET24a were purchased from Novagen (Madison, WI). The expression vector pIJ4070 was kindly provided by M. J. Bibb (The Johns Innes Institute, Norwich, UK). pL8, a genomic library clone containing clavulanic acid gene cluster (11), is maintained by the Dept. of Chemistry, The Johns Hopkins University (Baltimore, MD).

E. coli and *Streptomyces* plasmid DNA was isolated by standard methods (8) (17) and purified using the Genieprep DNA Isolation Kit (Ambion Inc.; Austin, TX). Genomic DNA from *S. clavuligerus* (ATCC 27064) and disruption mutants were isolated as described by Hopwood (8) and purified with the QIAamp Tissue Kit (Qiagen; Chatsworth, CA). Transformation of *E. coli* strains was preformed by standard procedures (17).

PCR amplification of *orf2* and its upstream region:

orf2 along with its 500-bp upstream regulatory region was amplified by PCR. Two primers (P5-1-2: AAGCTTATGGGGCCAGGCTATGCG [SEQ ID NO:1] and P3-2-2: GGATTCTCAGATGCTCAGGGCGC [SEQ ID NO:2]) were synthesized. The PCR reaction was carried in a 100 μl system containing 0.5 μg pL8 DNA, 0.2 nM of each primer, 0.2 mM dNTP, 10 μl DMSO and 1 × *Pfu* buffer. After heating for 5 min, 1 μl of *Pfu* DNA polymerase (2 U) (Stratagene, La Jolla, CA) was added. The PCR reaction was carried for 30 cycles, the conditions for the first 5 cycles were: 94 °C, 1 min; 55 °C, 1 min and 30 sec.; 72 °C, 1 min and 30 sec., then the annealing temperature was raised to 58 °C and 25 cycles were performed. In the last cycle, the elongation was carried out for 10 min to ensure the reaction was complete.

Transformation of *S. clavuligerus*.

The conditions for protoplast formation, regeneration, and DNA transformation were modified from the methods of Dominguez, Illing, and Malmberg (7) (9) (12). About 10^9 spores of wild-type or mutant *S. clavuligerus* were inoculated into 50 ml of TSB broth (14) in a 250-ml flask containing glass beads and grown at 26 °C with rotary shaking for 60 h. Mycelia were harvested by centrifugation, washed twice with 10.3% sucrose and once with P buffer [Tris-HCl, 0.31% (pH 8.0); $CaCl_2$ $2H_2O$ 0.368%; $MgCl_2$ $6H_2O$ 0.204%; sucrose

10%; glucose 1%]. The pellet was resuspended in P buffer containing 2 mg/ml lysozyme to the final volume of 10 ml and incubated at 30 °C for 25 min. The protoplast/mycelia mixture was filtered through a sterile cotton plug. The protoplasts were collected by centrifugation at 1000 × *g* for 10 min at 4 °C, washed three times with ice-cold P buffer and diluted to the final concentration of approximately 10^9/ml. Before DNA transformation, about 10^8 protoplasts were preheated in a 45 °C water bath for 10 min to inactivate the *S. clavuligerus* restriction system (2). The heat-treated protoplasts were transformed with 2 μg DNA and 500 μl of 25% (wt/vol) polyethylene glycol 1000 (NBS Biologicals, Hatfield, UK) solution was added immediately (8). After incubation at room temperature for 1 min, the transformed protoplasts were diluted with 2.5 ml ice-cold P buffer, collected by centrifugation, and resuspended in 1 ml P buffer. Each pre-dried R_2YEG regeneration plate (12) was plated with 100 μl transformed protoplasts and incubated at 26 °C. The plates were overlaid with 1.5 ml thiostrepton solution at the final concentration of 5 μg/ml or apramycin at 10 μg/ml.

Fermentation and analysis of clavulanic acid:

50 ml of TSB seed medium supplemented with glass beads was inoculated with either spores stock or from slants. 100 μg/ml apramycin was added when a strain containing a replicating plasmid was grown, while no antibiotic was added for growth of strains harboring an integrated plasmid. The seed culture was grown at 26 °C for 72 h at 300 rpm shaking. 0.5 ml seed culture was transferred to 50 ml SA+ fermentation medium in a 250 ml flask and incubated at 26 °C shaken as above for 120 or 144 h. 1 ml of culture was taken at 24, 48, 72, 96, 120 and 144 h and centrifuged at14000 rpm for 5 min.

Clavulanic acid was determined by the β-lactamase inhibition assay with *K. pneumoniae* subsp. *pneumoniae* and benzylpenicillin (15). Clavulanic acid was also detected by reaction with imidazole (6). Filtered fermentation supernatant was reacted with 0.25 equiv. vol. of 3 M imidazole reagent (pH 6.8) at 40°C for 20 min. The product of imidazole reaction showed a maximum absorbance at 312 nm (6) (16).

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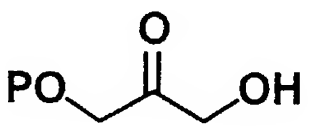
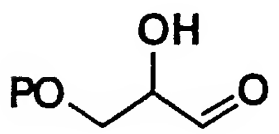
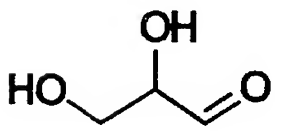
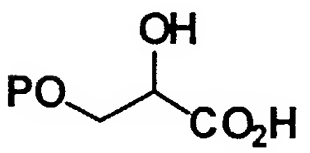
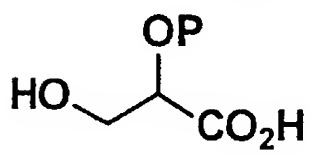
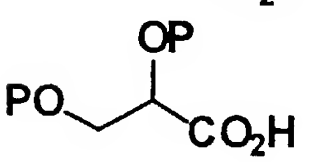
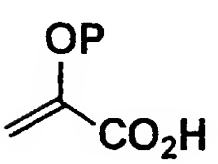
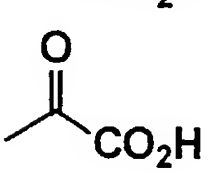
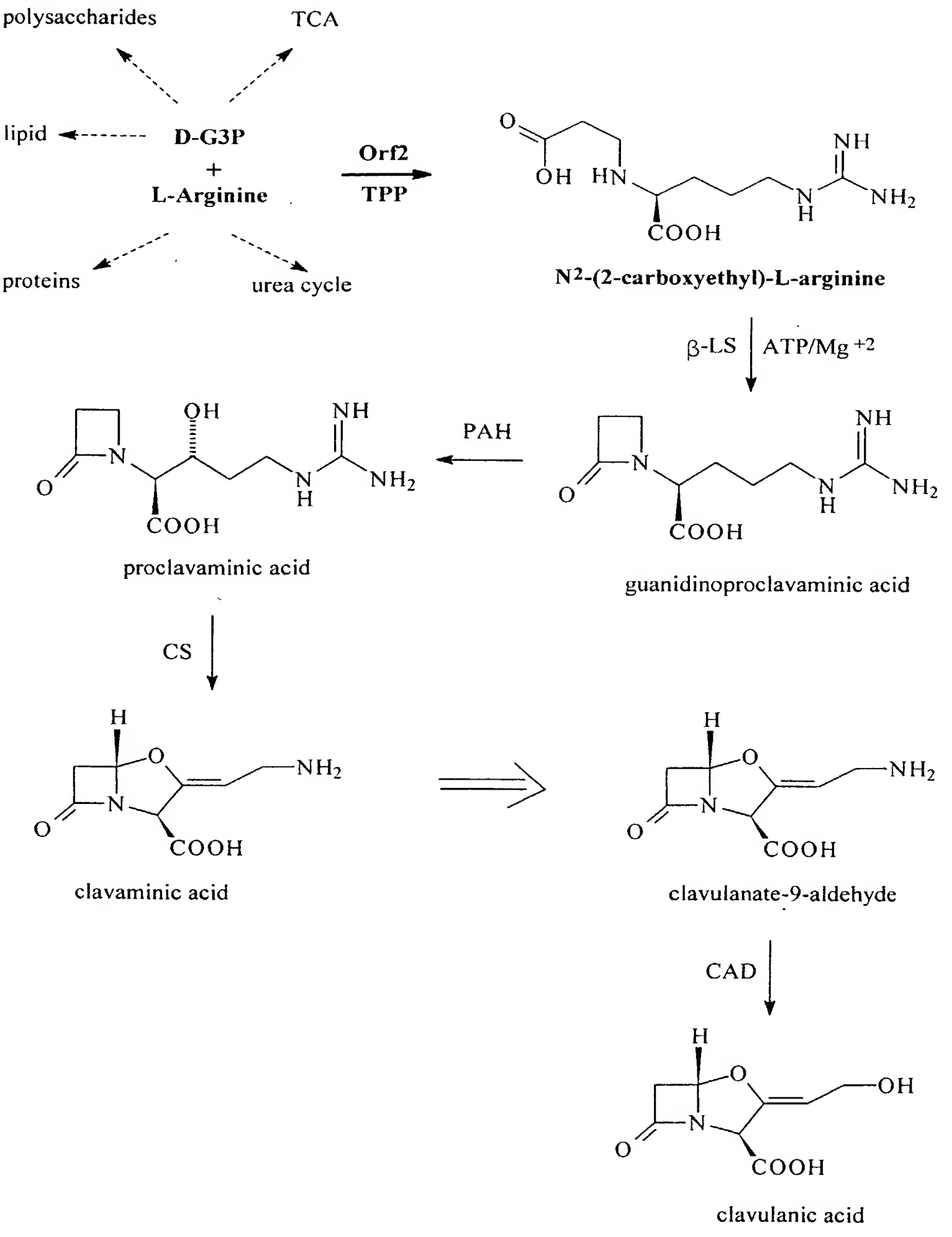
While the invention has been described in terms of its preferred embodiments, those skilled in the art will recognize that the invention can be practiced with modification within the spirit and scope of the appended claims. Accordingly, the present invention should not be

limited to the embodiments as described above, but should further include all modifications and equivalents thereof within the spirit and scope of the description provided herein.

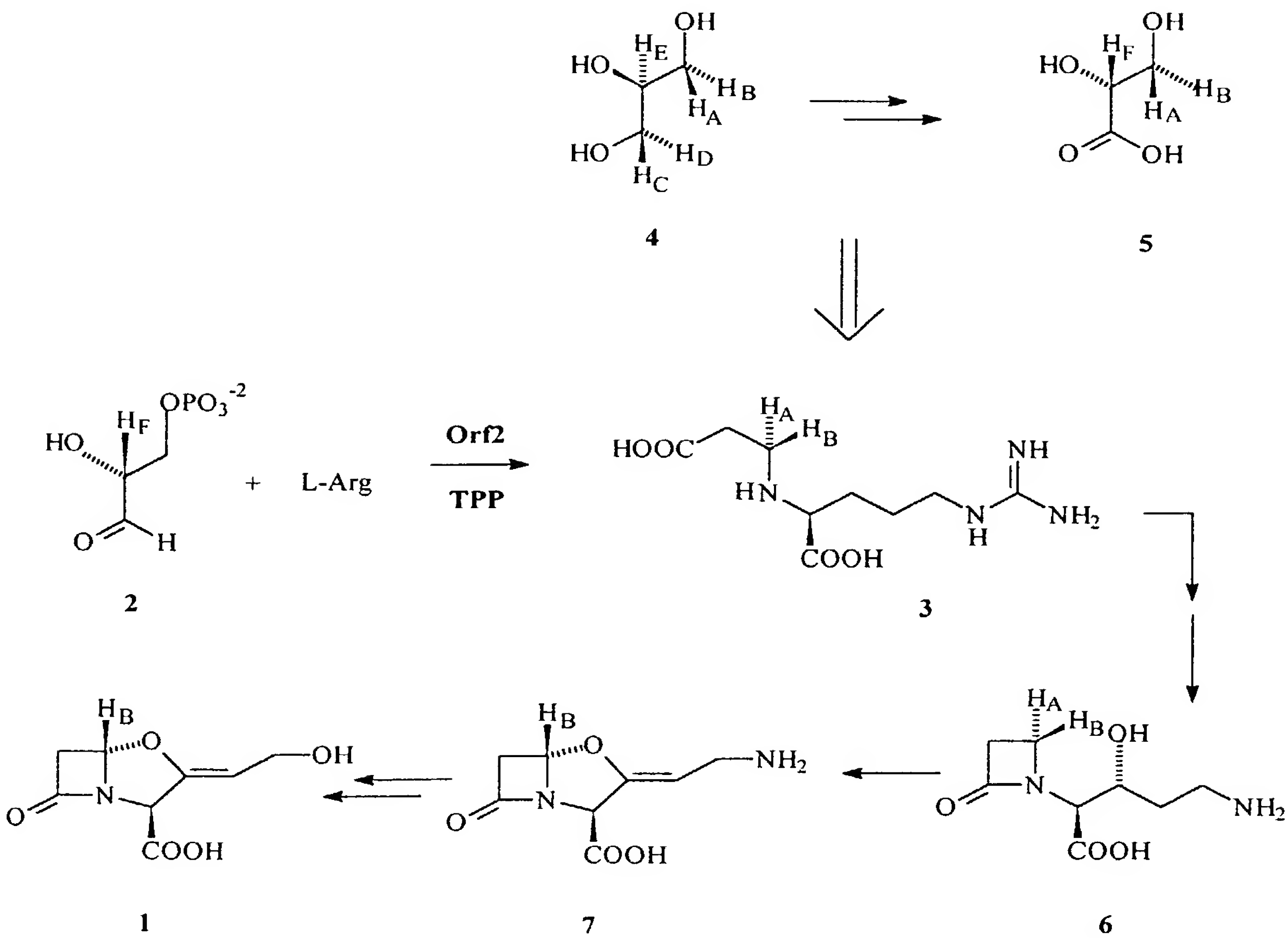
Table 1. Screening of potential C_3-carbohydrates with either a cell-free extract (CFE) of *E. coli* B834(DE3) overexpressing *orf2*, or dialyzed 30% ammonium sulfate pellet containing >95% CEA synthase.

structure	abbrev.	conc. (mM)	% conversion	
			CFE	30% $(NH_4)SO_2$ pellet
	DHAP	30	9.7	29.8
	D,L-G3P	30	3.1	3.9
		60	-	6.1
	D,L-GA	30	3.8	2.4
	3-PGA	30	3.5	0.5
	2-PGA	30	4.6	1.2
	2,3-DPGA	30	3.4	0.3
	PEP	30	2.8	2.6
	PA	30	3.4	1.1

Scheme 1.


polysaccharides
TCA
lipid
D-G3P
+
L-Arginine
Orf2
TPP
proteins
urea cycle
N2-(2-carboxyethyl)-L-arginine
β-LS
ATP/Mg +2
PAH
proclavaminic acid
guanidinoproclavaminic acid
CS
clavaminic acid
clavulanate-9-aldehyde
CAD
clavulanic acid

Scheme 2.


OH
HE
HO
HB
HA
HO
HD
HC
4
OH
HF
HO
HB
HA
O
OH
5
OPO3-2
HF
HO
O
H
2
+
L-Arg
Orf2
TPP
HA
HB
HOOC
HN
COOH
NH
N
H
NH2
3
HA
HB
OH
O
N
COOH
NH2
6
HB
O
NH2
O
N
COOH
7
HB
O
OH
O
N
COOH
1

We claim:

1. A method for increasing the production of clavulanic acid in a host comprising the step of:

increasing the level of N^2-(2-carboxyethyl)arginine synthase in said host, wherein said N^2-(2-carboxyethyl)arginine synthase catalyzes the condensation of L-arginine and D-glyceraldehyde-3-phosphate, resulting in increased production of clavulanic acid.

2. The method of claim 1 wherein said step of increasing is performed by gene dosing.

3. The method of Claim 2 wherein said increasing step is performed by providing said host with DNA encoding said N^2-(2-carboxyethyl)arginine synthase.

4. The method of claim 3 wherein said DNA is in a plasmid.

5. The method of claim 4 wherein said plasmid is a replicating plasmid.

6. The method of claim 5 wherein said replicating plasmid is pKC1139/pro-orf2-ter.

7. The method of claim 3 further comprising integrating said DNA into the chromosome of said host.

8. The method of claim 7 wherein said DNA is stably integrated via an integrative vector selected from the group consisting of pSET152/pro-orf2, pSET152/ermE(XbaI)-orf2 and pSET152/ermE(HindIII)-orf2.

9. The method of claim 3 wherein expression of said DNA is under the control of a constituitive promoter.

10. The method of claim 3 wherein said constitutive promoter is *ermE**.

11. The method of claim 1 wherein said increasing step is performed by adjusting fermentation conditions and/or providing additives which effect the optimization of N^2-(2-carboxyethyl)arginine synthase activity, wherein said optimization results in an increase in the production of clavulanic acid

12. The method of claim 1 wherein said host is *Streptomyces clavuligerus*.

13. A method for increasing the production of clavulanic acid in a host comprising the step of:

increasing the availability of precursors for reaction by N^2-(2-carboxyethyl)arginine synthase, wherein said step of increasing results in an increase in the production of clavulanic acid.

14. The method of claim 13 wherein said precursors are L-arginine and D-glyceraldehyde-3-phosphate.

15. The method of claim 13 wherein said host is *Streptomyces clavuligerus*.

16. The method of claim 13 wherein said increasing step is achieved by adjusting fermentation conditions and/or providing additives which optimize N^2-(2-carboxyethyl)arginine synthase activity.

17. A method for increasing the production of N^2-(2-carboxyethyl)arginine in a host cell, comprising,

enhancing a rate of condensation of L-arginine and D-glyceraldehyde-3-phosphate in said host cell, wherein said step of enhancing results in an increase in the production of N^2-(2-carboxyethyl)arginine in said host cell.

18. The method of claim 17 wherein said condensation of L-arginine and D-glyceraldehyde-3-phosphate is catalyzed by the enzyme N^2-(2-carboxyethyl)arginine synthase.

19. The method of claim 17 wherein said step of enhancing is carried out by increasing the copy number of a gene encoding N^2-(2-carboxyethyl)arginine synthase.

20. The method of claim 17 wherein said step of enhancing is carried out by adjusting fermentation conditions and/or providing additives which optimize N^2-(2-carboxyethyl) arginine synthase activity.

21. A method for preparing an composition having N^2-(2-carboxyethyl)arginine synthase activity, comprising the steps of

growing a culture of a host cell capable of synthesizing N^2-(2-carboxyethyl)arginine synthase,

harvesting and sonicating said culture,

removing cellular debris to produce a cellular supernatant,

fractionating said supernatant with ammonium sulfate to form a precipitated protein pellet,

resuspending said precipitated protein pellet to form a protein solution, and,

chromatographing said protein solution by affinity chromatography to isolate a

thiaminepyrophosphate-dependent enzyme having N^2-(2-carboxyethyl)arginine synthase activity.

22. The method of claim 21 wherein said affinity chromatography is carried out with an L-arginine agarose affinity column.

23. The method of claim 21 wherein said host is *Streptomyces clavuligerus*.

24. The method of claim 21 wherein said step of fractionating is carried out with 30% ammonium sulfate.

25. An assay for identifying substrates of the enzyme N^2-(2-carboxyethyl)arginine synthase, comprising the steps of

incubating a putative substrate with the enzyme N^2-(2-carboxyethyl)arginine synthase, thiaminepyrophosphate, and one known substrate of N^2-(2-carboxyethyl)arginine synthase, and

detecting the presence or absence of a condensation product of the putative substrate and the known substrate, wherein the presence of a condensation product is a positive result.

26. The assay of claim 25 wherein said known substrate is L-arginine.

27. The assay of claim 25 wherein said known substrate is D-glyceraldehyde-3-phosphate.

28. A host cell stably transformed with *orf2*.

29. The host cell of claim 28, wherein said host cell is *Streptomyces clavuligerus*.

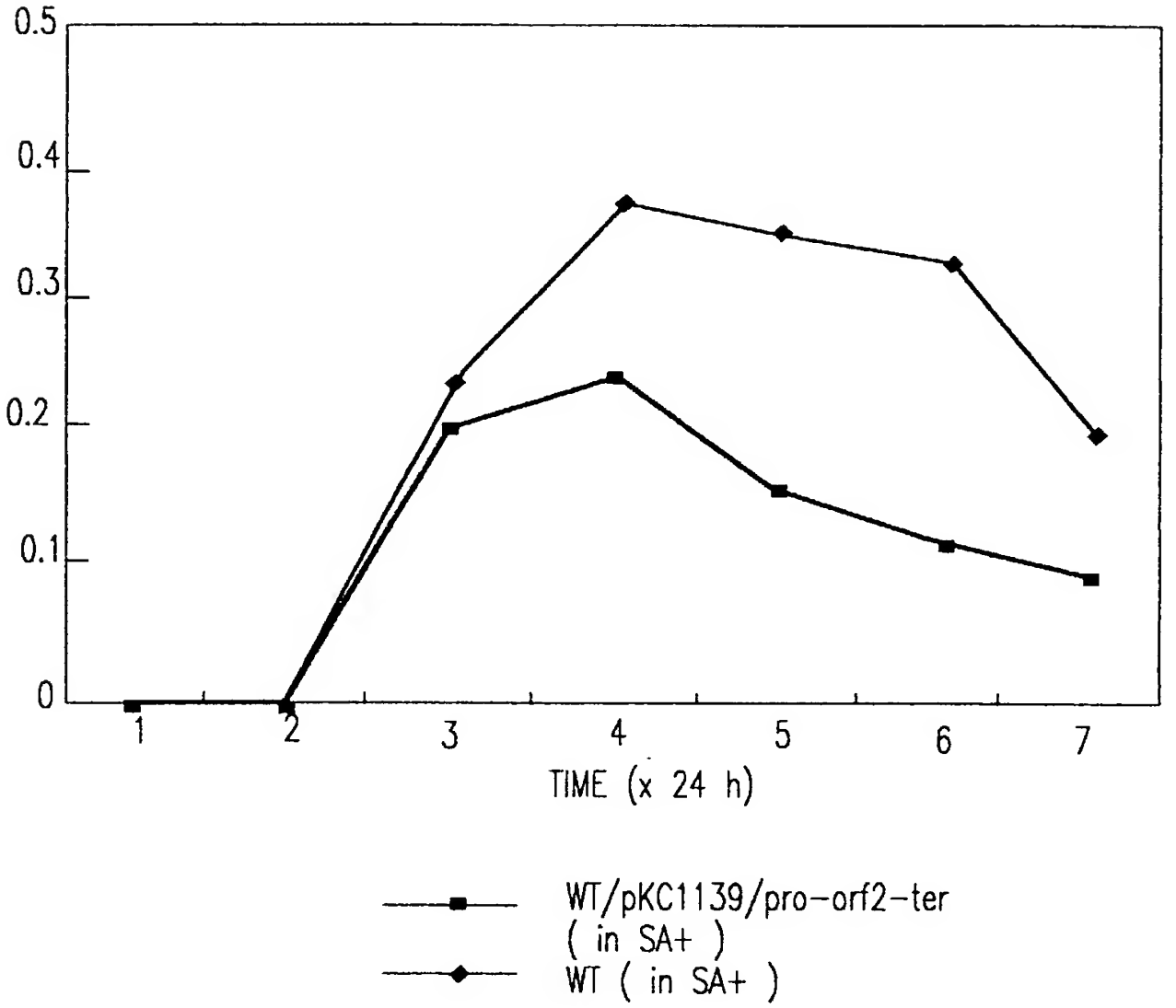
30. A condensation product of two substrates condensed by N^2-(2-carboxyethyl)arginine synthase.

ABSTRACT

The invention methods for the enhancement of clavulanic acid production. In particular, the invention provides a method for increasing the production of clavulanic acid by: gene dosage with *orf*2 from the clavulanic acid biosynthetic pathway in *Streptomyces clavuligerus*; and by manipulation of fermentation conditions, especially the concentration of DG3P, a substrate of N2(carboxyethyl)arginine synthase, the protein encoded by *orf*2. A method for preparing N2(carboxyethyl)arginine synthase is also provided, as is an assay for identifying its substrates.

PstI
BglII
HindIII
PstI
SalI
XbaI
BamHI
SacII
BssHII
EcoRV
EcoRI
SacI
XhoI
ClaI
SacI
KpnI
PstI
lacZ
pKC1139
6500 bp
rep ts
AmR
BglII
PstI
HindIII
SphI
SalI
XbaI
BamHI
SmaI
KpnI
SstI
EcoRI
BglII
SmaI
SmaI
SmaI
BclI
SmaI
BclI
essential
terminator
pIJ486
6200 bp
neo
tsr.
PstI
SmaI
PstI
BclI
BclI
SmaI/BglII
ter.
pT7Blue-3
BamHI
EcoRI
HindIII
SmaI
ClaI
NruI
ori
Kan
lacZ-
orf2 pro
pT7Blue-3/pro-orf2
Amp 5998 bp orf2
-lacZ
ScaI
BamHI
EcoRI
HindIII
PstI
PstI
BglII
HindIII
PstI
SalI
XbaI
BamHI/BglII
EcoRV/SmaI
EcoRI
ORI
rep ts
lacZ
pKC1139/ter
6982 bp
ter.
AmR
KpnI
ClaI
SacI
SacI
XhoI
HindIII
HindIII
PstI
BglII
oriT
PstI
rep ts
-lacZ-
pro
HindIII
EcoRI
EcoRI
KpnI
pKC1139/pro-orf2-ter
9082 bp orf2
ClaI
Am R
ter.
SacI
lacZ
XhoI
SacI
EcoRV/SmaI
EcoRI
EcoRI
HindIII
PstI
SalI
XbaI
BamHI/BglII
E. coliJM110
S.clavuligerusATCC27064

FIG.1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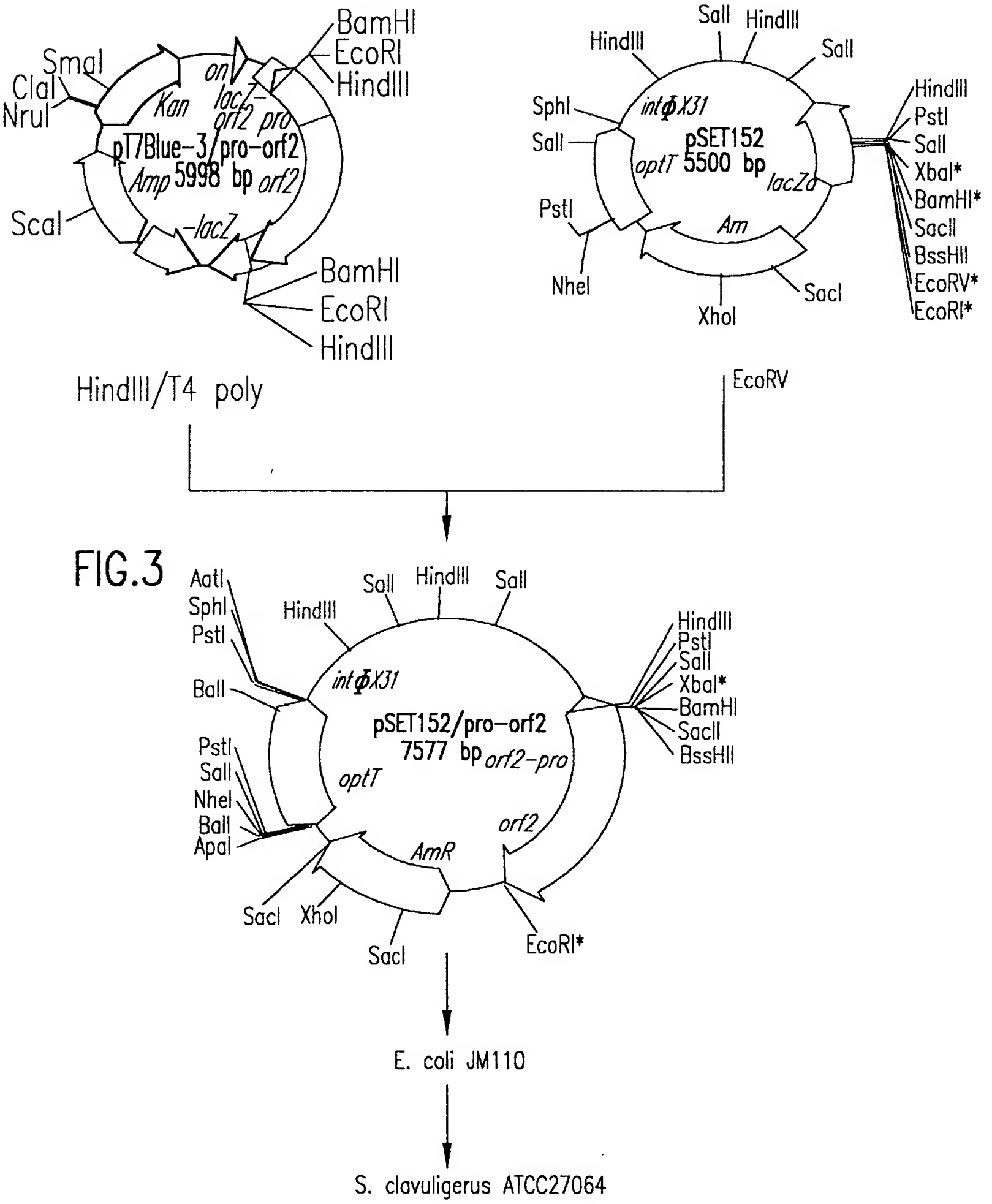
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6
7
TIME (x 24 h)
WT/pKC1139/pro-orf2-ter
(in SA+)
WT (in SA+)

FIG.2

FIG.3


SmaI
ClaI
NruI
ScaI
BamHI
EcoRI
HindIII
Kan
ori
lacI
orf2
pro
pT7Blue-3/pro-orf2
Amp 5998 bp
orf2
lacZ
BamHI
EcoRI
HindIII
HindIII/T4 poly
SalI
HindIII
HindIII
SalI
SphI
SalI
PstI
NheI
XhoI
SacI
HindIII
PstI
SalI
XbaI*
BamHI*
SacII
BssHII
EcoRV*
EcoRI*
intΦX31
pSET152
5500 bp
optT
lacZα
Am
EcoRV
AatI
SphI
PstI
BalI
PstI
SalI
NheI
BalI
ApaI
HindIII
SalI
HindIII
SalI
intΦX31
pSET152/pro-orf2
7577 bp orf2-pro
optT
AmR
orf2
SacI
XhoI
SacI
EcoRI*
HindIII
PstI
SalI
XbaI*
BamHI
SacII
BssHII
E. coli JM110
S. clavuligerus ATCC27064

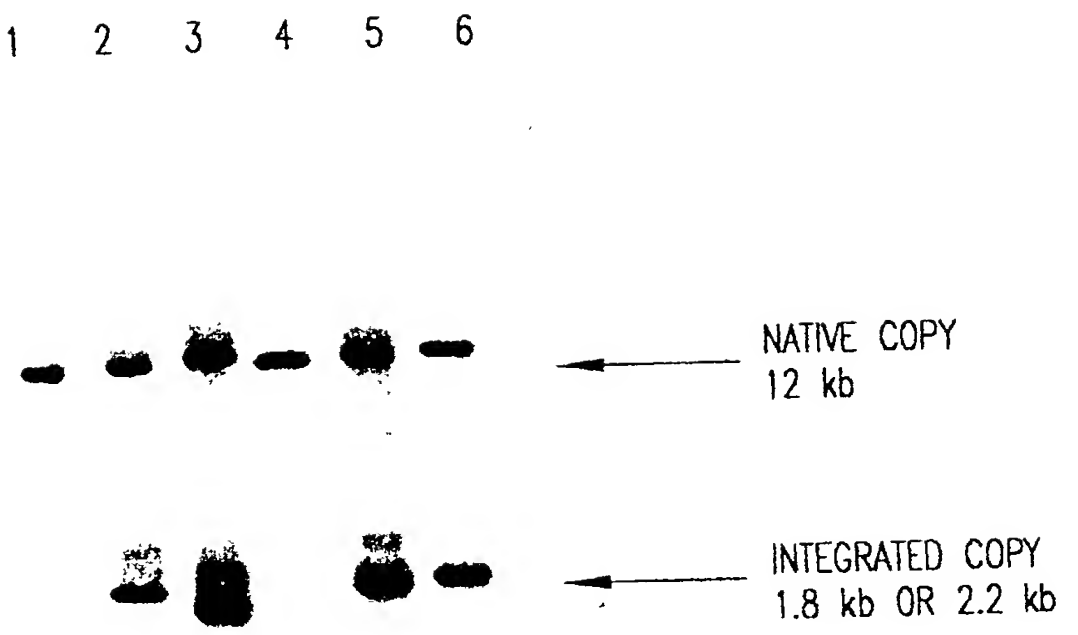
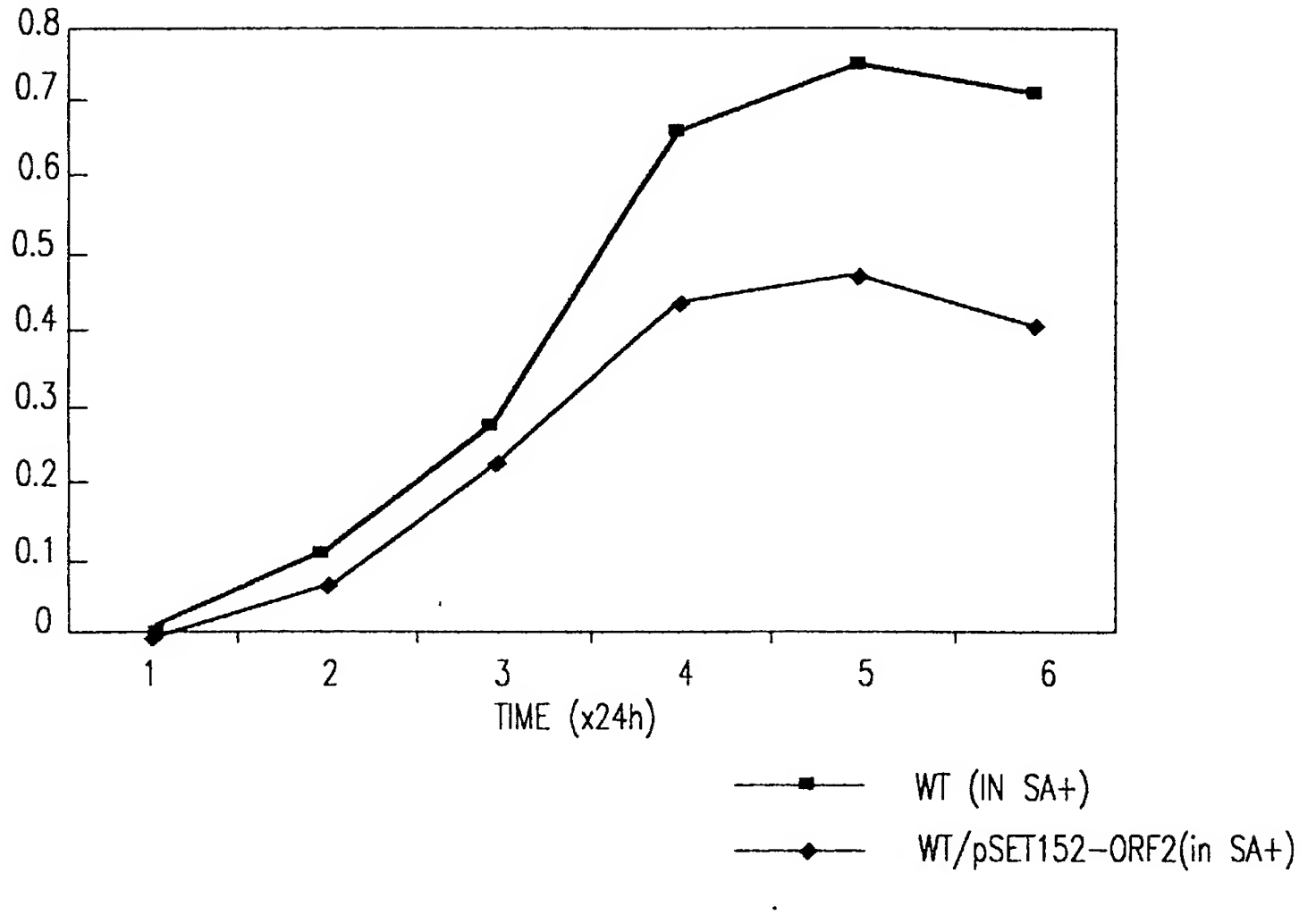


FIG.4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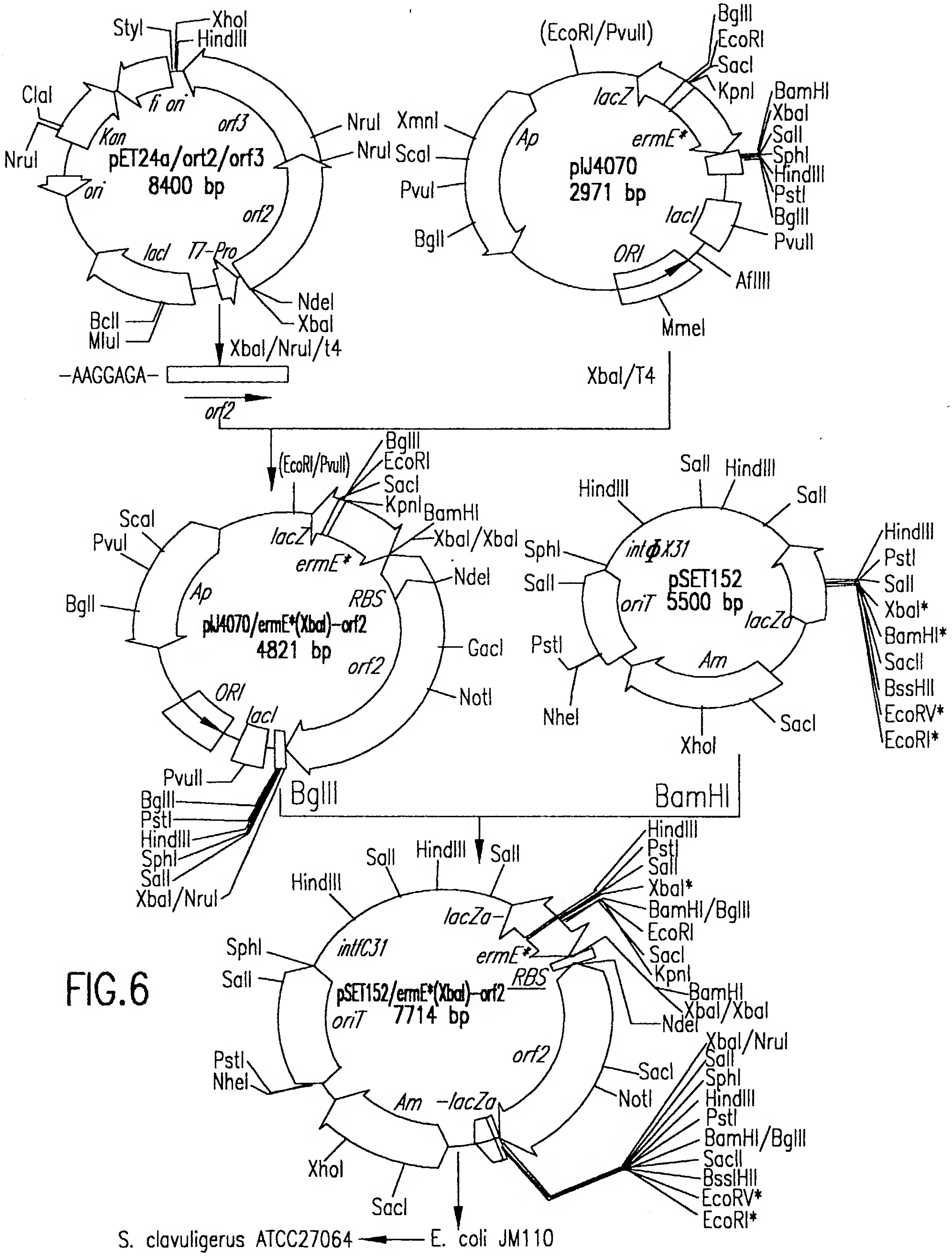
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6
TIME (x24h)
WT (IN SA+)
WT/pSET152-ORF2(in SA+)

FIG.5



StyI
XhoI
HindIII
ClaI
NruI
fi ori
orf3
Kan
pET24a/ort2/orf3
8400 bp
ori
orf2
lacI
T7-Pro
NruI
NruI
NdeI
XbaI
BclI
MluI
XbaI/NruI/t4
-AAGGAGA-
orf2
(EcoRI/PvuII)
BglII
EcoRI
SacI
KpnI
BamHI
XbaI
SalI
SphI
HindIII
PstI
BglII
PvuII
AflIII
MmeI
lacZ
ermE*
Ap
pIJ4070
2971 bp
lacI
ORI
XmnI
ScaI
PvuI
BglI
XbaI/T4
(EcoRI/PvuII)
ScaI
PvuI
BglI
lacZ
ermE*
Ap
RBS
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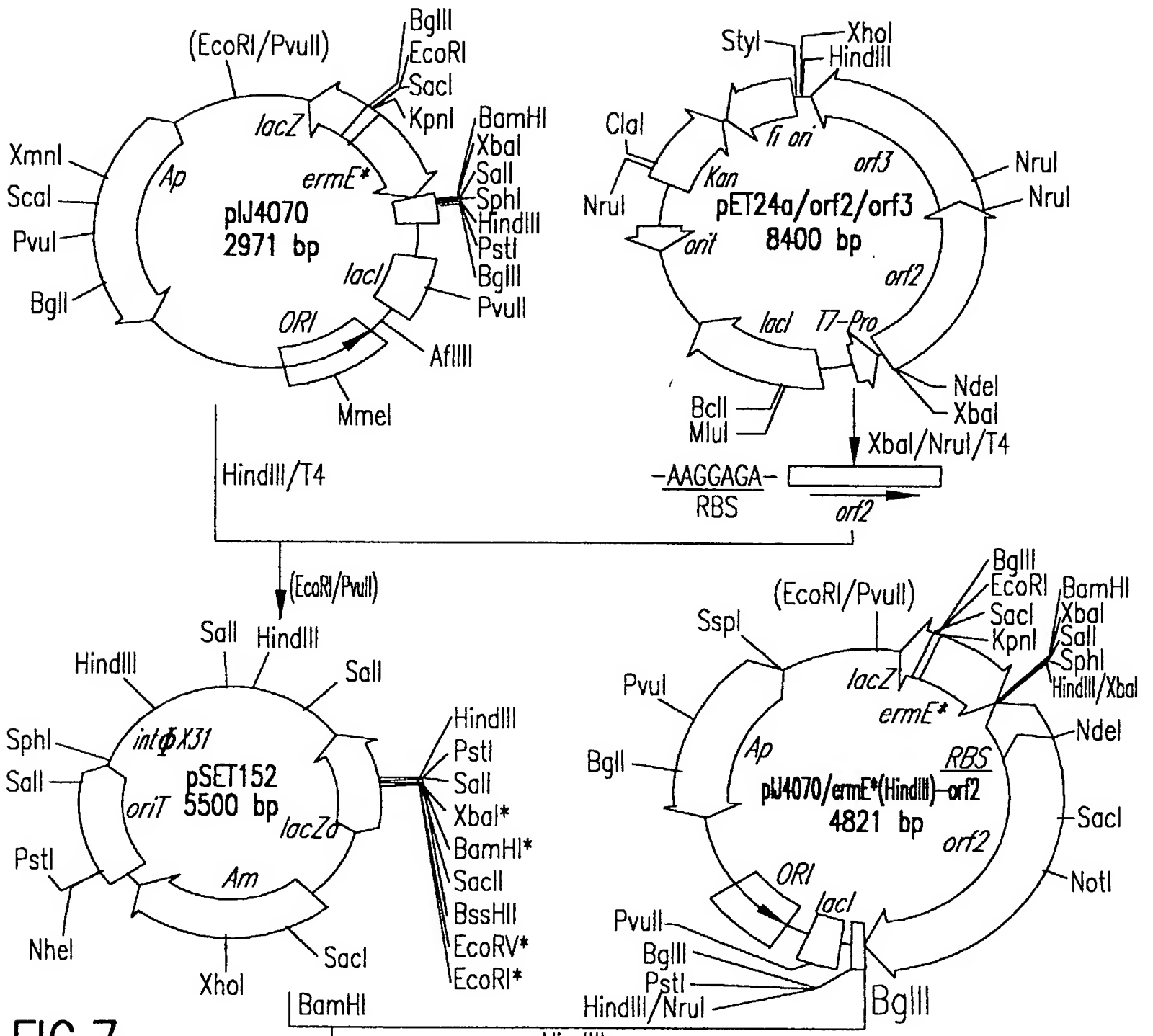
orf2
ORI
lacI
BamHI
XbaI/XbaI
NdeI
GacI
NotI
PvuII
BglII
PstI
HindIII
SphI
SalI
XbaI/NruI
BglII
SalI
HindIII
HindIII
SalI
SphI
SalI
intΦX31
pSET152
5500 bp
oriT
lacZa
Am
PstI
NheI
XhoI
SacI
HindIII
PstI
SalI
XbaI*
BamHI*
SacII
BssHII
EcoRV*
EcoRI*
BamHI
HindIII
SalI
HindIII
SalI
SphI
SalI
lacZa-
intfC31
ermE*
RBS
pSET152/ermE*(XbaI)-orf2
7714 bp
oriT
orf2
Am
-lacZa
PstI
NheI
XhoI
SacI
HindIII
PstI
SalI
XbaI*
BamHI/BglII
EcoRI
SacI
KpnI
BamHI
XbaI/XbaI
NdeI
XbaI/NruI
SalI
SphI
HindIII
PstI
BamHI/BglII
SacII
BssHII
EcoRV*
EcoRI*
SacI
NotI
S. clavuligerus ATCC27064
E. coli JM110

FIG.6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BglII
EcoRI
SacI
KpnI
BamHI
XbaI
SalI
SphI
HindIII
PstI
BglII
PvuII
AflIII
MmeI
lacZ
Ap
ermE*
lacI
ORI
pIJ4070
2971 bp
XmnI
ScaI
PvuI
BglI
HindIII/T4
StyI
XhoI
HindIII
ClaI
fi on
orf3
Kan
NruI
pET24a/orf2/orf3
8400 bp
ori
orf2
lacI
T7-Pro
NdeI
XbaI
BclI
MluI
XbaI/NruI/T4
-AAGGAGA-
RBS
orf2
(EcoRI/PvuII)
SalI
HindIII
SphI
intφX31
pSET152
5500 bp
oriT
lacZα
Am
PstI
NheI
XhoI
SacI
HindIII
PstI
SalI
XbaI*
BamHI*
SacII
BssHII
EcoRV*
EcoRI*
(EcoRI/PvuII)
SspI
PvuI
BglI
lacZ
ermE*
Ap
RBS
pIJ4070/ermE*(HindIII)-orf2
4821 bp
orf2
ORI
lacI
PvuII
BglII
PstI
HindIII/NruI
BglII
BglII
EcoRI
SacI
KpnI
BamHI
XbaI
SalI
SphI
HindIII/XbaI
NdeI
SacI
NotI
BamHI
HindIII/XbaI
SalI
XbaI
BamHI
HindIII
PstI
SalI
XbaI*
BamHI/BglII
HindIII/NruI
PstI
SacI
NotI
NdeI
SphI
KpnI
SacI
BglII
EcoRI
BamHI/BglII
SacII
BssHII
EcoRV*
EcoRI*
BamHI
HindIII
SalI
HindIII
SalI
lacZα
intfC31
SphI
PstI
pSET152/ermE*(HindIII)-orf2
7714 bp
oriT
orf2
PstI
NheI
AmR
RBS
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SacI
XhoI
SacI
E. coli JM110
S. clavuligerus ATCC27064

FIG.7

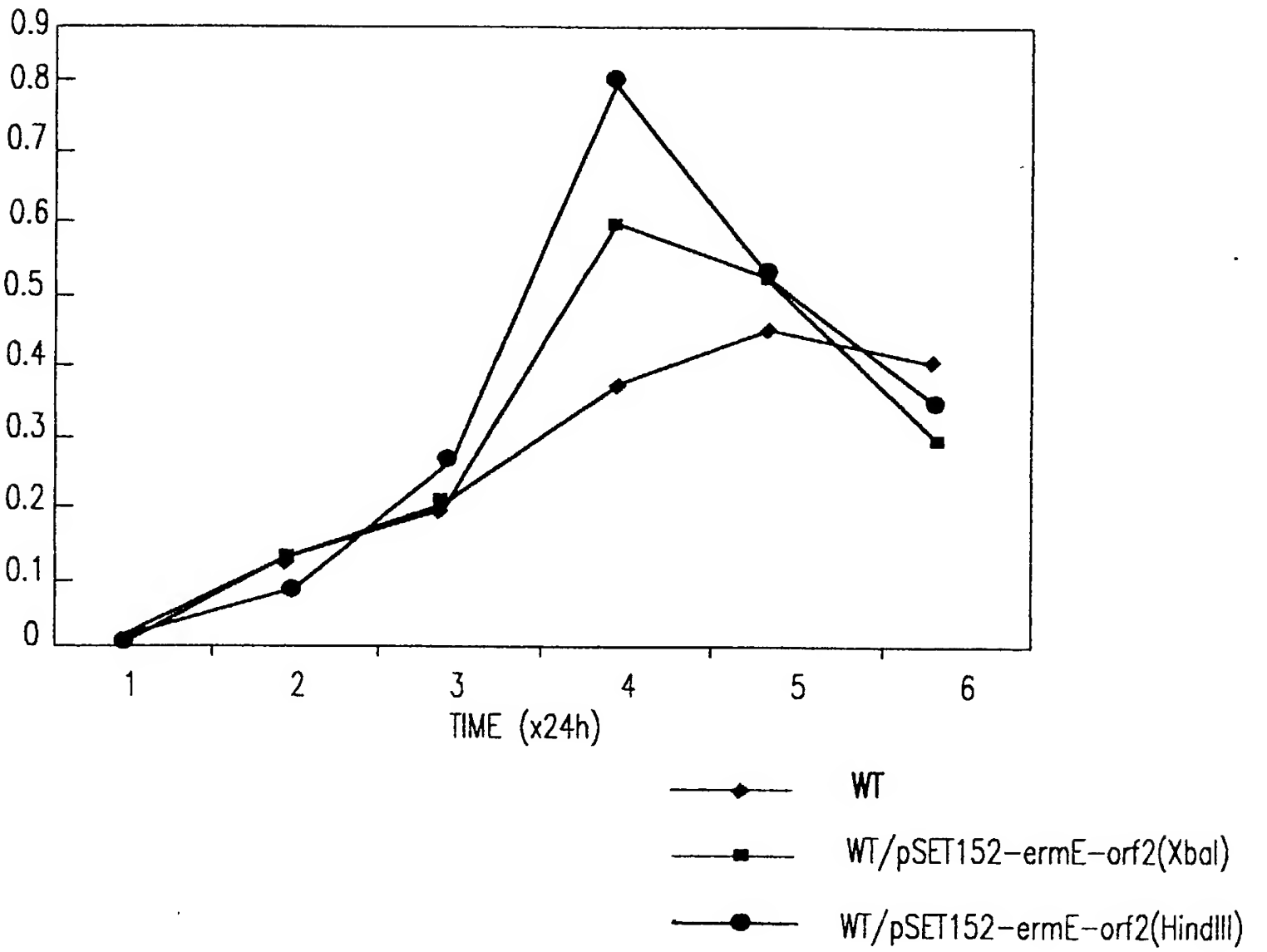
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6
TIME (x24h)
WT
WT/pSET152-ermE-orf2(XbaI)
WT/pSET152-ermE-orf2(HindIII)

FIG.8

FROM :Office Of Technology Transfer FAX NO. :4105167811 Apr. 23 2002 10:07AM P2/3

Application for United States Patent

DECLARATION AND POWER OF ATTORNEY

PTO JUN 26 2002 TRADEMARK OFFICE 1098

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name;

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

IMPROVEMENT OF CLAVULANIC ACID PRODUCTION

the specification of which:

(check one)

☒ is attached hereto

☐ was filed on_____, as Application Serial No._____ and was amended on_____. (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56*

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)

			priority claimed	
PCT/US00/25186 (Number)	PCT (Country)	16 September 1999 (Day/Month/Year Filed)	X yes	__ no
_____ (Number)	_____ (Country)	_____ (Day/Month/Year Filed)	__ yes	__ no
_____ (Number)	_____ (Country)	_____ (Day/Month/Year Filed)	__ yes	__ no

I hereby claim the benefit under Title 35, United States Code, § 119e of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

60/154,213 (Application Serial No.)	09/16/99 (Filing Date)	Pending (Status: patented, pending, abandoned)
_____ (Application Serial No.)	_____ (Filing Date)	_____ (Status: patented, pending, abandoned)

Power of Attorney: As a named inventor, I hereby appoint Michael E. Whitham, Reg. No. 32,635, Marshall M. Curtis, Reg. No. 33,138, Clyde R Christofferson, Reg. No. 34,138, C. Lamont Whitham, Reg. No. 22,424, Ruth E. Tyler-Cross, Reg. No. 45,922 and Olga V. Merkolouva, Reg. No. 48,757 as attorneys and/or agents to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. All correspondence should be directed to Whitham, Curtis & Christofferson, PC, 11491 Sunset Hills Road, Suite 305, Reston, Virginia 20190. Telephone calls should be directed to Whitham, Curtis & Christofferson, PC at (703) 787-9400. Faxes should be directed to 703-787-7557.

[redacted]

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1 - 00

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Inventor's Signature _____ Date: _____

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*Title 37, Code of Federal Regulations, § 1.56:

(a) A patent by its very nature is affected with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith toward the Patent and Trademark Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is canceled or withdrawn from consideration, or the application becomes abandoned.

(b) Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and (1) it establishes , by itself or in combination with other information, a prima facie case of unpatentability; or (2) it refutes, or is inconsistent with, a position the applicant takes in: (i) opposing an argument of unpatentability relied on by the Office, or (ii) asserting an argument of patentability.

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or First Inventor: Craig A. Townsend

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SEQUENCE LISTING

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Townsend, Craig
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